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Diethyl pyrocarbonate, a histidine-modifying agent, directly stimulates activity of ATP-sensitive potassium channels in pituitary GH_3 cells

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Abbreviations:
DEPC, diethyl pyrocarbonate
K_{ATP}-channel, ATP-sensitive K^+ channel
$[Ca^{2+}]_i$, intracellular Ca^{2+}
$[Ca^{2+}]_{ER}$, Ca^{2+} in the endoplasmic reticulum
erg, ether-à-go-go-related gene
I–V, current–voltage

ABSTRACT

The ATP-sensitive K^+ (K_{ATP}) channels are composed of sulfonylurea receptor and inwardly rectifying K^+ channel (Kir6.2) subunit. These channels are regulated by intracellular ADP/ATP ratio and play a role in cellular metabolism. Diethyl pyrocarbonate (DEPC), a histidine-specific alkylating reagent, is known to modify the histidine residues of the structure of proteins. The objective of this study was to determine whether DEPC modifies K_{ATP}-channel activity in pituitary GH_3 cells. Steady-state fluctuation analyses of macroscopic K^+ current at −120 mV produced power spectra that could be fitted with a single Lorentzian curve in these cells. The time constants in the presence of DEPC were increased. Consistent with fluctuation analyses, the mean open time of K_{ATP}-channels was significantly increased during exposure to DEPC. However, DEPC produced no change in single-channel conductance, despite the ability of this compound to enhance K_{ATP}-channel activity in a concentration-dependent manner with an EC_{50} value of 16 μM. DEPC-stimulated K_{ATP}-channel activity was attenuated by pretreatment with glibenclamide. In current–clamp configuration, DEPC decreased the firing of action potentials in GH_3 cells. A further application of glibenclamide reversed DEPC-induced inhibition of spontaneous action potentials. Intracellular Ca^{2+} measurements revealed the ability of DEPC to decrease Ca^{2+} oscillations in GH_3 cells. Simulation studies also demonstrated that the increased conductance of K_{ATP}-channels used to mimic DEPC actions reduced the frequency of spontaneous action potentials and fluctuation of intracellular Ca^{2+}. The results indicate that chemical modification with DEPC enhances K_{ATP}-channel activity and influences functional activities of pituitary GH_3 cells.

1. Introduction

The K_{ATP}-channels, which were originally discovered in heart [1], are distributed in many tissues, including pancreatic β-cells, cardiac myocytes, skeletal and smooth muscle cells, brain, neurons, and pituitary cells [2–4]. The activity of these channels plays crucial roles in various tissues by linking cell metabolism to electrical activity, because they are sensitive to inhibition by intracellular ATP [1,4]. These channels can be involved in the regulation of hormone secretion; vascular smooth tone and cellular response to ischemic stress in heart and brain [4].

K_{ATP}-channels are hetero-octamers consisting of two subunits, i.e. the pore-forming subunits of inwardly rectifying

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K^+ channel (Kir6.x) and regulatory subunits-sulphonylurea receptors (SUR) [5,6]. Kir6.x subunits, including Kir6.1 and Kir6.2, possess four ATP-binding sites [6,7], whereas SUR subunits, including SUR1, SUR2A and SUR2B, confer high-affinity block by sulphonylureas (e.g. glibenclamide) and stimulation by K_{ATP}-channel openers [8]. Different molecular forms of SUR and Kir6.x proteins can co-assemble to form K_{ATP}-channels with different functional and pharmacological properties in various tissues. Loss of function mutations in both Kir6.2 and SUR1 genes have been reported to produce congenital hyperinsulinism [9].

Diethyl pyrocarbonate (DEPC) is a known histidine-modifying reagent because it is a strong electrophile that reacts with the imidazole of histidine at neutral pH and forms an *N*-carbethoxyhistidyl derivative [10]. This compound has been previously utilized to demonstrate the importance of histidine residues in the function of different proteins including a variety of enzymes, transporters and ion channels [10–15]. In rat PC-12 neuroendocrine cell line, peptide transport was found to be inhibited by DEPC [16]. In human pituitary tissue, the activity of glutaminyl cyclase was modified with treatment of this compound [17]. Previous study also demonstrated that DEPC could modify the gating of *erg* K^+ current in neuronal NG108-15 cells [13]. A recent report also showed the ability of this compound to inhibit the discharge of intrapulmonary chemoreceptors in vivo [18].

In the present study, we sought to investigate whether DEPC could modify the activity of K_{ATP}-channels in pituitary GH_3 lactotrophs which can secrete prolactin and growth hormone. This cell line, in addition to the presence of voltage-dependent Ca^{2+} and K^+ currents, is known to exhibit large-conductance Ca^{2+}-activated K^+ and K_{ATP}-channels [3,19]. The results show that DEPC can stimulate K_{ATP}-channel activity and modify the firing of action potentials and $[Ca^{2+}]_i$ fluctuations. The study from a simulation model used to mimic electrical properties of GH_3 cells can also duplicate experimental observations, indicating that increased maximal conductance of K_{ATP}-channels affects spike discharge and change in $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Cell culture

GH_3 (a cell line from a rat anterior pituitary) cells were obtained from the Bioresource Collection and Research Center. Cells were cultured in 50 ml Ham's F-12 medium that was supplemented with 10% fetal calf serum and 2 mM L-glutamate in a 5% CO_2 atmosphere. Cells were subcultured once a week, and a new stock line was generated form frozen cells (frozen in 10% glycol in medium plus serum) every 3 months. The experiments were performed after 5 or 6 days of subcultivation (60–80% confluence) [19].

2.2. $[Ca^{2+}]_i$ measurements

Cells were loaded with 3 μM fura-2/AM [18] for 30 min at 25 °C in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Subsequently, cells were washed to remove non-hydrolyzed fura-2/AM. The glass coverslips on which the cells were grown were mounted in a 1 ml capacity plastic chamber and placed on an inverted fluorescence microscope (Olympus). Changes in $[Ca^{2+}]_i$ were monitored with digital imaging using a TILLvisION imaging system equipped with a Polychrome II high-speed monochromator (TILL Photonics). Fura-2 was excited sequentially by 340 and 380 nm light delivered from a xenon lamp via a 40×, 1.3 NA UV fluor oil objective (Olympus). Fluorescence images were collected at 510 nm at a rate of 0.5–2 frame/s by a Peltier-cooled charge-coupled camera. The ratio of fluorescence, R (340/380 nm) obtained from individual cell, was analyzed with the aid of TILLvisION software 4.0 (TILL Photonics) [20].

2.3. Electrophysiological measurements

Immediately before each experiment, GH_3 cells were dissociated with trypsin/EDTA solution and aliquot of cell suspension was placed into a recording chamber affixed to the stage of an inverted phase-contrast microscope. Cells were bathed at room temperature (20–25 °C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Ion currents were recorded in whole-cell, cell-attached or inside-out configuration of the patch-clamp amplifier (RK-400, Biologic) [19]. Patch pipettes (3–5 MΩ in bathing solution) were made from borosilicate glass capillary tubes using a two-step pipette puller (PP-830, Narishige), and the tips were heat-polished with a microforge (MF-83, Narishige). Drugs were applied by perfusion or added to the bath to obtain the final concentration indicated.

The signals, consisting of voltage and current tracings, were displayed on a digital storage oscilloscope (model HM507, Hameg Instruments). Currents were low-passed filtered at 1 kHz. A digidata 1322A interface (Axon Instruments) was used for the analog-to-digital/digital-to-analog conversion. Ion currents were taken at a sampling frequency of 10 kHz and stored without leakage correction and analyzed using the pClamp software (Axon Instruments), the Origin 7.5 software (Microcal Software Inc.) or custom-made macros in Excel (Microsoft).

The power spectra for macroscopic K^+ currents were smoothed by adjacent point averaging and then fitted with Lorentzian curves of the form $S(f) = S(0)/[1 + (f/f_c)2] = S(0)/[1 + (2\pi f\tau)^2]$, where $S(f)$ is power as a function of frequency, $S(0)$ the extrapolated power at frequency = 0 Hz, f_c the frequency at which power is $S(0)/2$, and τ is a time constant defined as $1/(2\pi f_c)$ which is related but not precisely equal to the mean open time. Current fluctuations were collected and calculated in the absence and presence of DEPC with the use of a Levenburg–Marquardt least-squares algorithm.

2.4. Single-channel analysis

Single-channel currents of K_{ATP}-channels were analyzed in the pCLAMP 9.0 software (Axon Instruments). Multi-gaussian adjustments of the amplitude distributions among channels were used to determine single-channel currents. The functional independence between the channels was verified by comparing the observed stationary probabilities. The open probability was evaluated using an iterative process to minimize the χ^2 calculated with a sufficiently large number of independent observations.

Open or closed lifetime distributions were fitted with logarithmically scaled bin width by using the method of McManus et al. [21]. To estimate all transition rates between states, single-channel data were idealized and converted to an ASCII format (i.e. a dwell-time file format) using the QUB software suite that was downloaded via the web site (http://www.qub.buffalo.edu). The data were then used to determine single-channel kinetic parameters by means of a maximum likelihood algorithm [22]. The highest log likelihood for the observed activity of K_{ATP}-channels in the control (i.e. in the absence of DEPC) was obtained with the gating scheme:

$$C_1 \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} C_2 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} O,$$

where O is open state, and C_1 and C_2 represent the closed states. On the basis of this linear scheme, the single-channel data were modeled, and transition rates were obtained using the QUB. This type of kinetic analysis was based on the assumption that the channel can be described by a finite-state Markovian model and that the channels in the patch are mutually independent.

To calculate the percentage increase in K_{ATP}-channel activity resulting from the presence of DEPC, the potential was held at −60 mV and the bath medium contained 100 μM ATP. DEPC at the different concentrations was applied to bath in detached patches of GH_3 cells. The probability of channel openings measured during exposure to DEPC (1 mM) was considered to be 100%. The concentration-dependent relation to DEPC on the stimulation of K_{ATP}-channel activity was fitted to the Hill equation by using a nonlinear regression analysis. That is:

$$\text{percentage increase} = \frac{E_{max}[C]^n}{EC_{50}^n + [C]^n},$$

where [C] represents the concentration of DEPC; EC_{50} and n are the concentrations required for a 50% inhibition and the Hill coefficient, respectively and E_{max} is DEPC-induced maximal stimulation of K_{ATP}-channels.

All values are reported as means ± S.E.M. The paired or unpaired Student's *t*-test and ANOVA with a least-significance difference method for multiple comparisons were used for the statistical evaluation of differences among means. Differences between the values were considered statistically significant when P was <0.05.

2.5. Modeling

Spontaneous action potentials and $[Ca^{2+}]_i$ oscillations were simulated using Ca^{2+}-based phantom bursting for pancreatic β-cells [23], because pituitary GH_3 cells are known to share similar electrical properties to those cells [3,19]. The basic model is essentially equivalent to the Chay–Keizer model and consists of a Ca^{2+} current, a delayed rectifier K^+ current, a Ca^{2+}-activated K^+ current, and a ATP-sensitive K^+ current [24]. Dynamic changes in $[Ca^{2+}]_i$ were further incorporated by Bertram and Sherman [23]. Simulations were carried out using the stochastic Euler algorithm as implemented in the program *xpp* [25] with the aid of the X-Win32 version of XPPAUT. Source files adapted in this study can readily be downloaded from http://mrb.niddk.nih.gov/sherman/gallery/beta/Biophan. Expressions and parameters for ion currents, equilibrium functions, and parameter values are modified in this study. In addition, *erg* K^+ current was incorporated to this model [26], given that it is consistently present in pituitary GH_3 cells [27]. Source file for *erg* K^+ current is available in http://senselab.med.yale.edu/senselab/modeldb. In the present simulations, the conductance values and reversal potentials, together with other parameters, used to solve the set of differential equations are listed in Table 1.

Table 1 – Default parameter values used for the modeling of pituitary GH_3 cells

Symbol	Description	Value
C_m	Membrane capacitance	5300 fF
g_{Ca}	Ca^{2+} current conductance	1000 pS
g_K	K^+ current conductance	1400 pS
g_{KCa}	Ca^{2+}-activated K^+ conductance	900 pS
g_{IR}	*erg* K^+ current conductance	5 pS
g_{KATP}	ATP-sensitive K^+ current conductance	500 pS
V_{Ca}	Ca^{2+} reversal potential	50 mV
V_K	K^+ reversal potential	−75 mV

2.6. Drugs and solutions

DEPC, a viscous liquid of density 1.12 g/ml, was purchased from Sigma and stored desiccated at 5 °C. Appropriate amounts of the liquid were added to the bath immediately before use. In water, DEPC hydrolyzes rapidly to ethanol and CO2 with a half-life of 9–24 min at 25 °C [10]. Glibenclamide, iberiotoxin and paxilline were obtained from Alomone Labs. Azimilide was a gift from Proctor and Gamble Pharmaceuticals.

The composition of normal Tyrode's solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES–NaOH buffer 5.5 (pH 7.4). To record membrane potential, the patch pipettes were filled with solution (in mM): KCl 140, KH_2PO_4 1, $MgCl_2$ 1, EGTA 0.1, Na_2ATP_3 1, Na_2GTP 0.1, and HEPES-KOH buffer 5 (pH 7.2). In the cell-attached or inside-out configuration of single-channel recordings, high K^+ bathing solution contained (mM): KCl 145, $MgCl_2$ 0.53 and HEPESKOH buffer 5 (pH 7.4), and the pipette solution contained (mM): KCl 145, $MgCl_2$ 2, and HEPES–KOH buffer 5 (pH 7.2). For single-channel recordings, high K^+-bathing solution contained (in mM) KCl 145, $MgCl_2$ 0.53, and 5 mM HEPES–KOH 5 (pH 7.4) and pipette solution contained (in mM): KCl 145, $MgCl_2$ 2, and HEPES–KOH 5 (pH 7.2).

3. Results

3.1. Effect of DEPC on membrane currents in pituitary GH_3 cells

In the first series of experiments, macroscopic K^+ currents in these cells were examined. Cells were bathed in Ca^{2+}-free Tyrode's solution and the holding potential was set at the level of −120 mV. This current was sensitive to inhibition by glibenclamide (10 μM), yet not by paxilline (1 μM) or iberiotoxin (200 nM), suggesting that ATP-sensitive K^+ current was present in GH_3 cells [3]. When cells were exposed to DEPC

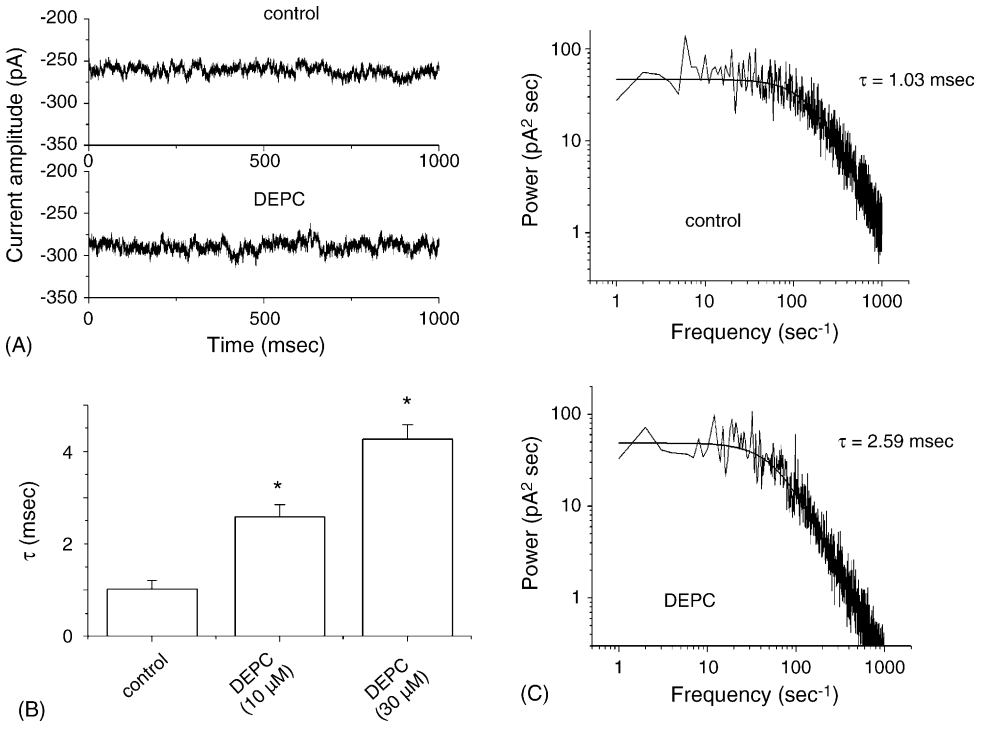

Fig. 1 – Effect of DEPC on membrane currents and steady-state fluctuation analysis in the absence and presence of DEPC. In these experiments, cells were bathed in Ca^{2+}-free Tyrode's solution. The cell was held at the level of −120 mV. (A) Current traces obtained in control and during exposure to DEPC (30 μM). Each trace was acquired at a steady-state level of current. (B) Power spectra obtained in the absence and presence of DEPC (30 μM). Power spectra are shown with superimposed fitted single Lorentzian curves. (C) Bar graph showing the effect of DEPC on the time constant obtained from power spectra which were measured at −120 mV. Each point represents the mean ± S.E.M. (N = 6–10). * Significantly different from control.

(30 μM), current amplitude was increased from 255 ± 15 to 298 ± 26 pA (N = 8). In addition, an increase in "noise" for currents was also observed in the presence of DEPC (Fig. 1A). In the first approach, the kinetic properties of macroscopic K^+ currents were determined by steady-state fluctuation analysis. The power spectra of current fluctuations were then calculated and fitted with Lorentzian curves of the form as described in Methods. As shown in Fig. 1B, single Lorentzian curves obtained in the absence and presence of DEPC (30 μM) provided excellent fits to the power spectra. The resulting corner frequency was higher after treatment with DEPC (30 μM), suggesting the underlying channels have a longer mean open time in the presence of this compound. The time constants of the fitted Lorentzian curves were significantly increased at −120 mV when cells were exposed to 10 and 30 μM DEPC (Fig. 1C). If one assumes that channel gate independently, it is possible to extract information about the underlying kinetics from spectral density analysis of macroscopic current fluctuations. These results thus suggest that chemical modification with DEPC influence the behavior of macroscopic K^+ currents in GH_3 cells.

3.2. *DEPC enhances K_{ATP}-channel activity in single-channels recordings measured from GH_3 cells*

In order to investigate whether DPEC can alter the activity of K_{ATP}-channels, single-channel recordings were further performed. In these experiments, GH_3 cells were bathed in symmetrical K^+ concentration (145 mM) and bath medium contained 100 μM ATP. In inside-out configuration, each cell was held at the level of −60 mV. When DPEC (30 μM) was applied to the bath, channel activity was greatly increased (Fig. 2). The presence of DPEC (30 μM) significantly increased the probability of channel openings from 0.0014 ± 0.0012 to 0.0145 ± 0.0031 (N = 8). After washout of DEPC, the channel activity almost returned to the control. When the intracellular surface of detached patches was pre-exposed to glibenclamide, DEPC-stimulated channel activity was significantly reduced (Fig. 2B). However, further application of DEPC (30 μM) was not found to have any effect on the open probability after membrane patches were exposed to 1 mM ATP. The results suggest that DEPC can alter ATP-sensitivity of K_{ATP}-channels.

The relationship between the concentration of DEPC and the probability of channel openings is shown in Fig. 2C. This compound increases K_{ATP}-channel activity in a concentration-dependent manner with an EC_{50} value of 16 μM. However, no change in single-channel conductance of K_{ATP}-channels in the presence of DEPC was demonstrated (Fig. 2D). Consistent with previous observations [3], the single-channel conductance calculated with a linear I–V relationship in control was 78 ± 5 pS (N = 6). The value of single-channel conductance remained unaltered in the presence of 30 μM DEPC (79 ± 4 pS, N = 5).

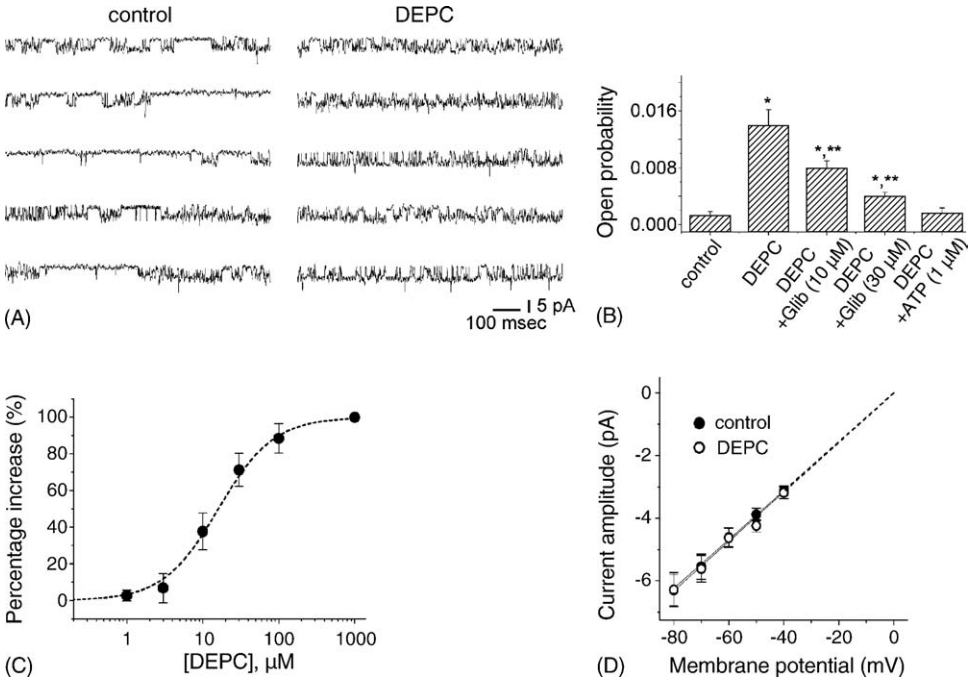

Fig. 2 – Effect of DEPC on the activity of K_{ATP}-channels in GH_3 cells. The experiments were conducted with symmetrical K^+ concentration (145 mM). Under inside-out configuration, the holding potential was set at −60 mV and bath solution contained 100 μM ATP. (A) The activity of K_{ATP}-channels recorded before (left) and during exposure to 30 μM DEPC (right). Downward deflections indicate the opening events of the channel. (B) Effects of DEPC (30 μM) on K_{ATP}-channel activity in the absence and presence of glibenclamide. Potential was held at −60 mV. In the experiments with DEPC plus glibenclamide, DEPC was added after application of glibenclamide. Glib, glibenclamide. In these experiments, bath medium contained 100 μM ATP. However, in those with DEPC plus ATP (1 mM), DEPC was added when detached patches were bathed in 1 mM ATP. *Significantly different from control. **Significantly different from DEPC alone group. Each point represents mean ± S.E.M. (N = 5–7). (C) Concentration–response curve for DEPC-induced stimulation of K_{ATP}-channel activity. Each detached patch was held at −60 mV. Bath medium contained 100 μM ATP. The open probability in the presence of 1 mM DEPC was taken to be 100% and those obtained at different concentrations (1–100 μM) of DEPC was compared (mean ± S.E.M.; N = 4–8 for each point). The dashed line is the fit of the data with a Hill function. The values for EC_{50}, maximally inhibited percentage of K_{ATP}-channel activity, and the Hill coefficient were 16 μM, 99%, and 1.2, respectively. (D) Averaged I–V relations of K_{ATP}-channels obtained in the absence (filled circles) and presence (open circles) of DEPC (10 μM). Each point represents the mean ± S.E.M. (N = 4–8). Notably, single-channel conductance obtained in the absence and presence of DEPC is nearly identical.

3.3. *Effect of DEPC on mean open time of K_{ATP}-channel in pituitary GH_3 cells*

The effect of DEPC on the gating of these channels was further analyzed because fluctuation analyses of macroscopic K^+ currents showed a longer time constant after application of this compound. In an excised patch of control cell, open time histogram at the level of −60 mV can be fitted by a one exponential curve (Fig. 3A). The time constant for the open time histogram was 1.78 ± 0.12 ms (N = 5). Interestingly, the application of DEPC to the bath significantly increased the time constant of the open state (Fig. 3B). For example, DEPC at a concentration of 30 μM increased the mean open time to 2.62 ± 0.23 ms (N = 5). These results demonstrate that, in agreement with the analyses from macroscopic currents, DEPC can prolong the mean open time of K_{ATP}-channels.

In order to gain more insight on the channel kinetics, single-channel currents in the absence and presence of DEPC were idealized and modeled using the kinetic scheme described in Section 2. The gating scheme consists of one open and two closed states. Transition rates between states were derived from maximum likelihood estimations [22]. Fig. 4 shows the examples of simulated single-channel data that were generated using transitional rates of K_{ATP}-channels obtained in the absence and present of DEPC. The equilibrium dissociation constants in the absence and presence of DEPC (30 μM) were estimated to be 0.47 ± 0.04 and 1.09 ± 0.07, respectively (N = 5), while equilibrium gating constants in the absence and presence of DEPC (30 μM) were 0.056 ± 0.003 and 0.677 ± 0.015, respectively (N = 5). These results lead us to suggest that during exposure to DEPC, the difference in the gating constant (12-folds) is much greater than in the dissociation constant (two-folds).

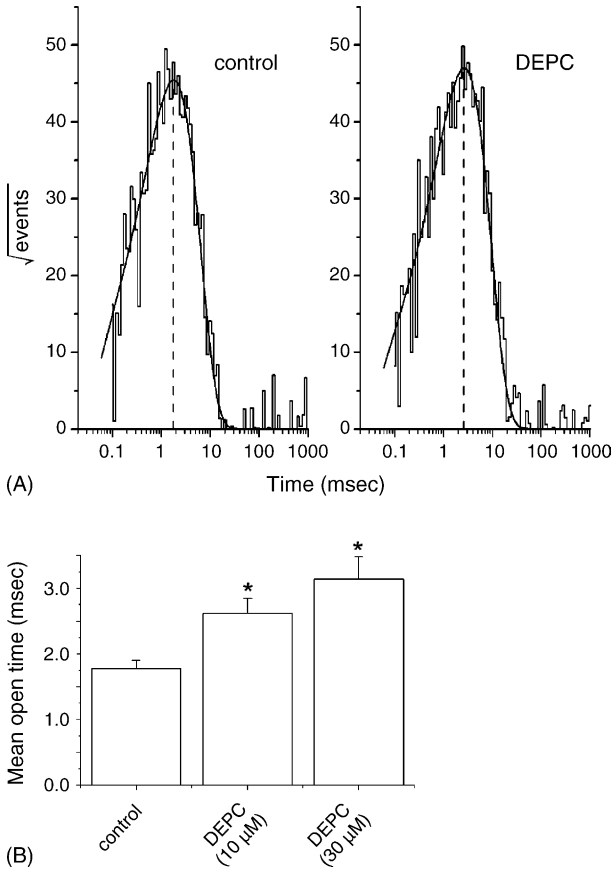

Fig. 3 – Effect of DEPC on mean open time of K_{ATP}-channels in GH_3 cells. Inside-out configuration was performed in these experiments and the potential was held at −60 mV. Cells were bathed in symmetrical K^+ solution (145 mM) and bath medium contained 100 μM ATP. (A) Open time histograms in the control (left side) and after application (right) of 30 μM DEPC. Of note, the abscissa and ordinate show the logarithm of open times (ms) and the square root of the number of events, respectively. The dashed line shown in each lifetime distribution is placed at the value of the time constant in open state. (B) Bar graph showing summary of effect of DEPC on mean open time in GH_3 cells. *Significantly different from control. Each bar indicates the mean ± S.E.M. (n = 5–6).

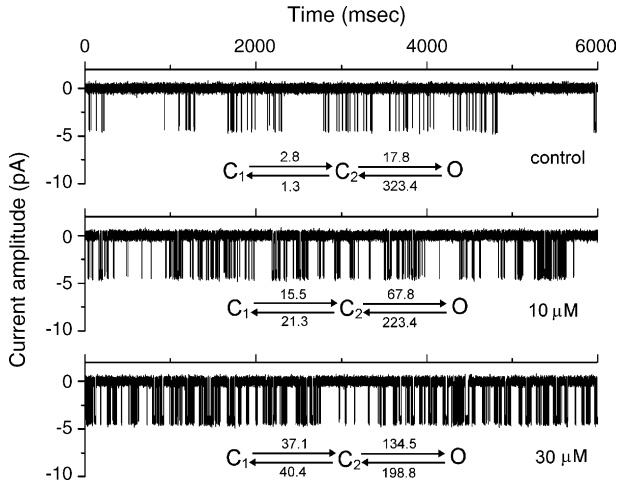

Fig. 4 – Simulated single-channel currents in the absence (upper) and presence of 10 and 30 μM DEPC. The kinetic models used to analyze the observed data measured at a holding potential of −60 mV are shown in the lower part of each simulated current. Each horizontal arrow pointing to the left represents the binding of DEPC or closing of a channel, whereas each arrow to the right represents the dissociation of DEPC or opening of a channel. The units are μM/s or/s. O, open state and C_1 and C_2, first and second closed states.

3.4. *Effect of DEPC on spontaneous action potentials in pituitary GH_3 cells*

In another series of experiments, the effect of DEPC on repetitive firing of action potentials was also investigated. In these experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Current–clamp configuration was performed with a K^+-containing pipette solution. The typical effect of DEPC (30 μM) on spontaneous action potentials in these cells is illustrated in Fig. 5. When cells were exposed to DEPC, membrane potential was gradually hyperpolarized and repetitive firing of action potentials was readily decreased. For example, DEPC (10 μM) significantly reduced the firing frequency to 0.89 ± 0.05 Hz (N = 5) from a control of 2.23 ± 0.11 Hz (N = 6). Glibenclamide (10 μM) significantly increased it to 1.54 ± 0.08 Hz (N = 5). However, azimilide (30 μM) did not have any effect on DEPC-induced decrease in repetitive firing of action potentials. The firing frequency between DEPC and DEPC plus azimilide was 0.88 ± 0.05 and 0.86 ± 0.06 Hz (N = 5). Azimilide is a blocker of *erg* K^+ currents [28]. It is thus clear that DEPC can regulate the firing of action potentials in pituitary GH_3 cells. DEPC-induced inhibition of spontaneous actions potentials is primarily mediated through its activation of K_{ATP}-channels, although this compound might affect the kinetics of *erg* K^+ currents [13].

3.5. *Effect of DEPC on $[Ca^{2+}]_i$ in pituitary GH_3 cells*

With the aid of intracellular Ca^{2+} measurements, we further examined whether DEPC could affect spontaneous $[Ca^{2+}]_i$ oscillations in GH_3 cells. As shown in Fig. 6, the frequency of spontaneous $[Ca^{2+}]_i$ spiking was reduced after application of DEPC (30 μM). When cells were exposed to DEPC (30 μM), the frequency of $[Ca^{2+}]_i$ fluctuations was significantly decreased to 0.9 ± 0.1 Hz from a control value of 2.3 ± 0.6 Hz (N = 7). In continued presence of DEPC, the application of glibenclamide (10 μM) significantly increase the frequency to 1.7 ± 0.4 (N = 5). However, further application of iberiotoxin (200 nM) had little or no effect on DEPC-induced decrease in the frequency of $[Ca^{2+}]_i$ fluctuations. Therefore, the effect of DEPC on spontaneous $[Ca^{2+}]_i$ oscillations present in GH_3 cells could be closely associated with the opening of a K_{ATP}-channel caused by this compound.

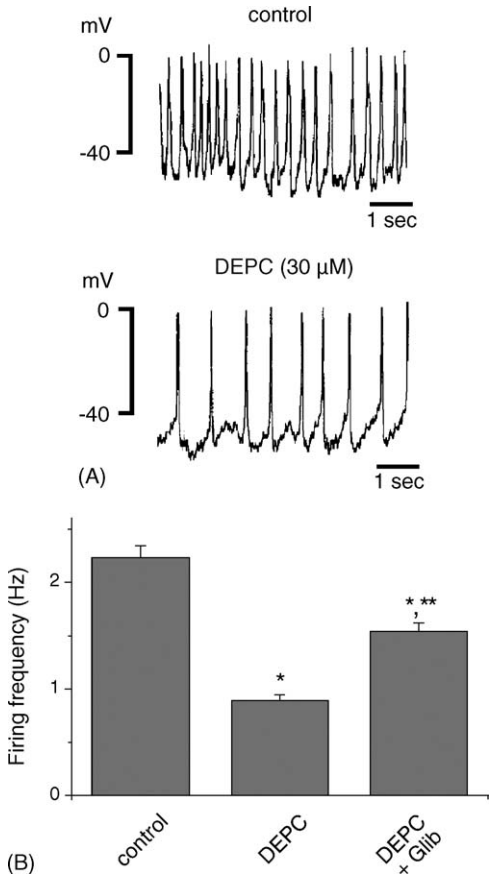

Fig. 5 – Effect of DEPC on the firing of action potentials in GH_3 cells. In these experiments, current–clamp conditions were performed. (A) potential traces obtained in the control and 2 min after application of DEPC (10 μM). (B) Bar graph showing the effect of DEPC (30 μM) and DEPC plus glibenclamide on the firing frequency in GH_3 cells. Glib, glibenclamide (10 μM).

3.6. *Effect of DEPC on spontaneous action potentials and $[Ca^{2+}]_i$ in modeled GH_3 cells*

Fig. 7 illustrates the time course of repetitive firing of action potentials and the changes in $[Ca^{2+}]_i$ simulated from a modified cell model. In the control, the firing frequency is 2.3 Hz. When the maximal conductance of K_{ATP}-channels

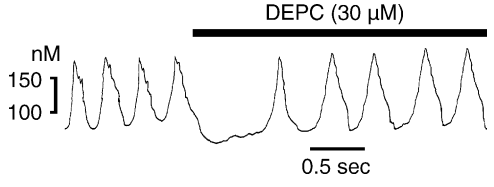

Fig. 6 – Effect of DEPC on repetitive changes in $[Ca^{2+}]_i$ in GH_3 cells. In these experiments, fura two-loaded cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Addition of DEPC (30 μM) indicated at the horizontal bar suppresses the frequency of spontaneous $[Ca^{2+}]_i$ oscillations.

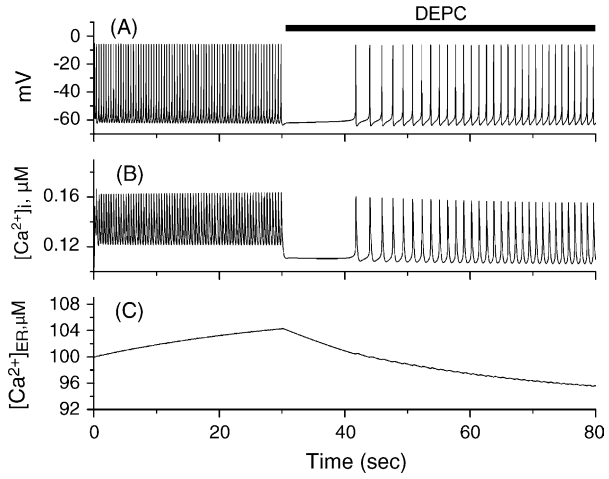

Fig. 7 – Effect of DEPC on simulated action potentials (A), $[Ca^{2+}]_i$ (B) and $[Ca^{2+}]_{ER}$ (C) in a cell model which mimics electrical behaviors of GH_3 cell. Application of DEPC (30 μM) as indicated at horizontal bar was mimicked by an increase of maximal conductance of K_{ATP}-channels from 500 to 530 pS.

was increased from 500 to 530 pS, the discharge rate was greatly declined to 0.9 Hz. This result closely resembled our experimental observations showing that application of DEPC (30 μM) reduced the firing frequency be approximately 60% (Fig. 5). Upon chemical modification with DEPC, the model cell remained hyperpolarized for about 10 s, followed by a decrease in the discharge. Furthermore, concomitant with changes in spontaneous action potentials, fluctuations of spontaneous $[Ca^{2+}]_i$ were also reduced after the conductance of K_{ATP}-channels was increased. Moreover, the $[Ca^{2+}]_{ER}$ was found to be gradually declined following changes in the conductance of K_{ATP}-channels. It is thus anticipated that the K_{ATP}-channel is an important target of modification by DEPC with which repetitive firing of action potentials, along with fluctuations of $[Ca^{2+}]_i$, can be altered in pituitary GH_3 lactotrophs.

4. Discussion

The major findings of the present study are as follows. First, in pituitary GH_3 cells, DEPC, a specific modifier of histidine residues, altered the time constants of macroscopic K^+ currents. Second, DEPC increased the activity of K_{ATP}-channels in a concentration-dependent fashion with no change in single-channel conductance. Third, DEPC could increase mean open time of K_{ATP}-channels. Fourth, this compound reduced the firing of spontaneous action potential and the fluctuations of $[Ca^{2+}]_i$. Fifth, simulation model also predicted that the decreased maximal conductance of K_{ATP}-channels could duplicate the experimental results showing the ability of DEPC to increase K_{ATP}-channel activity. The present findings suggest that the underlying mechanism by which DEPC affects Ca^{2+} signaling in GH_3 cells is associated with its stimulation of K_{ATP}-channel activity. The effects on Ca^{2+} signaling and ion channels could be the important

mechanisms underlying DEPC-induced action, if similar results occur in neurons or endocrine cells in vivo [16,18,29].

In this study, concentration-dependent stimulation of K_{ATP}-channel activity with an EC_{50} value of 16 μM was observed in the presence of a histidine-modifying reagent named DEPC. These results suggested that histidine residue(s) were actively involved in K_{ATP}-channel activity. In addition, the presence of glibenclamide before chemical modification with DEPC could prevent the increased channel activity from DEPC-induced stimulation. Glibenclamide is a prototypical blocker of K_{ATP}-channels, which binds to the SUR subunit of the channel. Therefore, the binding ability of glibenclamide to block K_{ATP}-channels was not suppressed by DEPC modification, suggesting that essential histidine residues did not lie on the binding site of glibenclamide. However, in the presence of excessive amounts of ATP (1 mM), the stimulatory effect of DEPC was much weaker, suggesting that histidine(s) are present in ATP-binding domain on the intracellular leaflet of the channel. It is thus likely that intracellular ATP decreases the channel open probability by binding to a site consisting of histidine residue(s) which may participate in the gating of K_{ATP}-channels. The binding sites for DEPC involved in channel gating appear to be localized at the inner surface of the cell membrane. In addition, in current–clamp conditions, membrane was hyperpolarized and the firing of action potentials was gradually reduced by loading the cell via the patch pipette with 30 μM DEPC.

The increase in macroscopic K^+ currents caused by chemical modification with DEPC is apparently not explained by an increase in single-channel amplitude of K_{ATP}-channels, because no significant difference was found in single-channel conductance between the absence and presence of DEPC. Inability of this compound to modify single-channel conductance suggests that the increased responsiveness of the channel to this compound is secondary to the alterations remote from the pore region of the channels. We also found that DEPC produced a significant increase in mean open time of the channel which is consistent with the results from steady-state fluctuation analysis. These findings can primarily account for the increased probability of channel openings. More importantly, on the basis of the simple gating scheme described in Section 2, the mechanism of action of this compound on K_{ATP}-channel activity was further deduced. The results lead us to propose that the stimulatory actions of DEPC primarily lie on the gating of channels, because the magnitude of difference between the absence and presence of DEPC in the gating constant is much greater than that in the dissociation constant. Our findings also support the notion that the imidazole groups present in histidine residue(s) participate in the gating of K_{ATP}-channels. It still remains to be clarified how the binding events for this compound are functionally coupled to the opening of channel pores.

In sequence analysis of the Kir6.2 channel, seven histidine residues have been found (i.e. H70, H175, H186, H215, H276–H278) [5]. A previous report showed that the amino acids for ATP-binding sites of K_{ATP}-channel were K185 in the C-terminus and R50 in the N-terminus [30]. The N6 atom of the adenine ring for ATP molecule has been recently reported to interact with E179 and R301 in the Kir6.2 subunit [31]. The location of H175 is in close proximity to E179. The protonation of H175 present in a K_{ATP}-channel was found to modify channel activity [32]. Therefore, it is tempting to speculate that chemical modification with DEPC enhances K_{ATP}-channel activity primarily through the medication of H186 and/or H175 in the C-terminus. These two residues seem to have an important functional significance in regulation of K_{ATP}-channel activity. However, any modification with other histidine residues may also involve structural change, thereby causing an allosteric effect on nucleotide binding site in the protein. Nevertheless, DEPC may be an intriguing compound utilized for studying the gating of the K_{ATP}-channel.

The firing of action potentials present in GH_3 cells was reduced during exposure to DEPC. Unlike glibenclamide, azimilide, a blocker of *erg* K^+ channels, was found to have little or no effects on DEPC-induced inhibition of spontaneous discharge. The results suggest that the main effect of this compound on spontaneous action potentials in these cells is attributed to the stimulation of K_{ATP}-channels.

With the aid of a simulation model presented in this study, we also examined the effect of DEPC on repetitive firing of action potentials found in pituitary GH_3 cells. As a result, we are able to show clearly that the agents known to increase the conductance of K_{ATP}-channels (e.g. DEPC) can effectively hyperpolarize the cell and decrease the firing frequency, thereby leading to a reduction of $[Ca^{2+}]_i$ fluctuations. Taken together, DEPC-induced increase in K_{ATP}-channel activity may significantly contribute to its effect on the activity of neurons or endocrine cells in vivo.

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REFERENCES

[1] Noma A. ATP-regulated K^+ channels in cardiac muscle. Nature 1983;305:147–8.
[2] Bernardi H, De Weille JR, Epelbaum J, Mourre C, Amoroso S, Slama A, et al. ATP-modulated K^+ channels sensitive to antidiabetic sulfonylureas are present in adenohypophysis and are involved in growth hormone release. Proc Natl Acad Sci USA 1993;90:1340–4.
[3] Wu SN, Li HF, Chiang HT. Characterization of ATP-sensitive potassium channels functionally expressed in pituitary GH_3 cells. J Membr Biol 2000;178:205–14.
[4] Seino S, Miki T. Physiological and pathophysiological roles of ATP-sensitive K^+ channels. Prog Biophys Mol Boil 2003;81:133–76.
[5] Inagaki N, Gonoi T, Clement JP, Namba N, Inazawa J, Gonzalez G, et al. Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. Science 1995;270:1166–70.
[6] Clement JP, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar Bryan L, et al. Association and stoichiometry of K_{ATP}-channel subunits. Neuron 1997;18:827–38.

[7] Markworth E, Schwanstecher C, Schwanstecher M. ATP4$^-$ mediates closure of pancreatic beta-cell ATP-sensitive potassium channels by interaction with one of four identical sites. Diabetes 2000;49:1413–8.
[8] Mannhold R. K_{ATP}-channel openers: structure-activity relationships and therapeutic potential. Med Res Rev 2004;24:213–66.
[9] Dunne MJ, Cosgrove KE, Shepherd RM, Aynsley-Green A, Lindley KJ. Hyperinsulinism in infancy: from basic science to clinical disease. Physiol Rev 2004;84:239–75.
[10] Miles EW. Modification of histidyl residues in proteins by diethylpyrocarbonate. Meth Enzymol 1977;47:431–43.
[11] Yamasaki K, Daiho T, Saino T, Kanazawa T. Modification of histidine 5 in sarcoplasmic reticulum Ca^{2+}-ATPase by diethyl pyrocarbonate causes strong inhibition of formation of the phosphoenzyme intermediate from inorganic phosphate. J Biol Chem 1997;272:30627–36.
[12] Dzhandzhugazyan KN, Plesner L. Diethyl pyrocarbonate inactivates CD39/ecto-ATPDase by modifying His-59. Biochim Biophys Acta 2000;1466:267–77.
[13] Meves H. Slowing of ERG current deactivation in NG108-15 cells by the histidine-specific reagent diethylpyrocarbonate. Neuropharmacology 2001;41:220–8.
[14] Zhao K, Luo G, Zhao GM, Schiller PW, Szeto HH. Transcellular transport of a highly polar 3+ net charge opioid tetrapeptide. J Pharmacol Exp Ther 2003;304:425–32.
[15] Zhou F, Pan Z, Ma J, You G. Mutational analysis of histidine residues in human organic anion transporter 4 (hOAT4). Biochem J 2004;384:87–92.
[16] Hussain I, Zanic-Grubisic T, Kudo Y, Boyd CA. Functional and molecular characterization of a peptide transporter in the rat PC12 neuroendocrine cell line. FEBS Lett 2001;508:350–4.
[17] Bateman Jr RC, Temple JS, Misquitta SA, Booth RE. Evidence for essential histidines in human pituitary glutaminyl cyclase. Biochemistry 2001;40:11246–50.
[18] Pilarski JQ, Hempleman SC. Imidazole binding reagent diethyl pyrocarbonate (DEPC) inhibits avian intrapulmonary chemoreceptor discharge in vivo. Respir Physiol Neurobiol 2005 [Epub ahead of print].
[19] Wu SN, Li HF, Chiang HT. Stimulatory effects of δ-hexachlorocyclohexane on Ca^{2+}-activated K^+ currents in GH_3 lactotrophs. Mol Pharmacol 2000;57:865–73.
[20] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[21] McManus OB, Blatz AL, Magleby KL. Sampling, log bin, fitting, and plotting durations of open and shut intervals from single-channels and the effect of noise. Pfluegers Arch 1987;410:530–53.
[22] Qin F, Auerbach A, Sachs F. Estimating single-channel kinetic parameters from idealized patch-clamp data containing missed events. Biophys J 1996;79:264–80.
[23] Bertram R, Sherman. A calcium-based phantom bursting model for pancreatic islets. Bull Math Biol 2004;66:1313–44.
[24] Chay TR, Keizer J. Minimal model for membrane oscillations in the pancreatic β-cell. Biophys J 1983;42: 181–90.
[25] Ermentrout B. Simulating, analysing and animating dynamical system: a guide to XPPAUT for researchers and students. Philadelphia: Society for Industrial and Applied Mathematics; 2002.
[26] Chiesa N, Rosati B, Arcangeli A, Olivotto M, Wanke E. A novel role for HERG K^+ channels: spike-frequency adaptation. J Physiol 1997;501:313–8.
[27] Wu SN, Jan CR, Li HF, Chiang HT. Characterization of inhibition by risperidone of the inwardly rectifying K^+ current in pituitary GH_3 cells. Neuropsychopharmacology 2000;23:676–89.
[28] Busch AE, Eigenberger B, Jurkiewicz NK, Salata JJ, Pica A, Suessbrich H, et al. Blockade of HERG channels by the class III antiarrhythmic azimilide: mode of action. Br J Pharmacol 1998;123:23–30.
[29] Lu DC, Erlichman JS, Leiter JC. Diethyl pyrocarbonate (DEPC) inhibits CO_2 chemosensitivity in Helix aspersa. Respir Physiol 1998;111:65–78.
[30] Trapp S, Haider S, Jones P, Sansom MSP, Aschroft FM. Identification of residues contributing to the ATP binding site of Kir6.2. EMBO J 2003;22:2903–12.
[31] Antcliff JF, Haider S, Proks P, Sansom MSP, Aschroft FM. Functional analysis of a structural model of the ATP-binding site of the K_{ATP}-channel Kir6.2 subunit. EMBO J 2005;24:229–39.
[32] Xu H, Cui N, Yang Z, Wu J, Giwa LR, Abdulkadir L, et al. Direct activation of cloned K_{ATP}-channels by intracellular acidosis. J Biol Chem 2001;276:12898–902.